Alteration of Membrane Barrier in Stripped Rough Microsomes from Rat Liver on Incubation with GTP: Its Relevance to the Stimulation by this Nucleotide of the Dolichol Pathway for Protein Glycosylation

DANIÈLE GODELAINE, HENRI BEAUFAY, MAURICE WIBO, and ANNE-MARIE RAVOET
Laboratoire de Chimie Physiologique, Université de Louvain, Belgium; and International Institute of Cellular and Molecular Pathology, B-1200 Brussels, Belgium

ABSTRACT The membrane barrier of stripped rough microsomes from rat liver is markedly altered on incubation with GTP at 37°C: after 30 min the structure-linked latency of mannose-6-phosphatase was considerably reduced, and esterase and nucleoside diphosphatase were partly released into the suspension medium. This phenomenon was already maximal with 30 μM GTP and was specific for this nucleotide. Similar conditions enhance the dolichol-mediated glycosylation of protein in microsomes incubated with uridine diphosphate *N*-acetylglucosamine and guanosine diphosphate mannose (Godelaine, D., H. Beaufay, M. Wibo, and A. Amar-Costesec, 1979, *Eur. J. Biochem.*, 96:17–26; Godelaine, D., H. Beaufay, and M. Wibo, 1979, *Eur. J. Biochem.*, 96:27–34). The GTP-induced permeability and glycosylation activities evolved in parallel in rough microsomes subjected to various treatments to detach the ribosomes and were maximal after removal of ≃60% of the RNA. In addition, GTP had no effect of this type in smooth microsome subfractions. Triton X-100, in spite of complex inhibitory effects on glycosylation reactions, mimicked the action of GTP by increasing the amount of microsomal dolichylphosphate that reacts with uridine diphosphate *N*-acetylglucosamine and by enhancing synthesis of dolichylpyrophosphoryl-chitobiose at concentrations >2 mg/ml. Thus, GTP may activate dolichol-mediated glycosylation reactions in stripped microsomes by lowering the permeability barrier that prevents access of sugar nucleotides to the inner aspect of the membrane. The genuine role of GTP in the functioning of the endoplasmic reticulum membrane in situ remains unknown. Because GTP seems to act only on rough microsomes, we hypothesize that this role is somehow related to biosynthesis of protein by the rough endoplasmic reticulum.

Bound polysomes and the underlying endoplasmic reticulum (ER)[1] membrane act as a coordinated system for the synthesis of glycoproteins. The nascent peptides are vectorially discharged within the lumen or inserted into the membrane of the ER (for reviews, see 28, 36) and core-glycosylated synchronously. Protein *N*-glycosylation involves a number of enzymes and lipid intermediates firmly bound to the ER membrane (for reviews, see 33, 40). It is coupled to the transport of sugar moieties across the membrane, from the cytoplasmic compartment where the sugar nucleotide precursors are synthesized (9), to the luminal compartment of ER where the saccharide chains of glycoproteins have been located (20, 23, 26, 35).

Previous studies from this laboratory have revealed striking effects of GTP on protein glycosylation in rough microsomes from rat liver (16–18). When rough microsomes are incubated with uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) and guanosine diphosphate mannose (GDP-Man) in the absence of detergent, all reactions but the first (doli-

[1] *Abbreviations used in this paper:* Dol-PP-GlcNAc, dolichylpyrophosphoryl-*N*-acetylglucosamine; Dol-PP-GlcNAc$_2$, dolichylpyrophosphoryl-chitobiose; Dol-PP-oligosaccharides, dolichylpyrophosphate-linked oligosaccharides; ER, endoplasmic reticulum; GDP-Man, guanosine diphosphate mannose; GMP-PCP, guanylyl(β,γ-methylene)-diphosphonate; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 AUGUST 1983 340–350
 · 0021-9525/83/08/0340/11 $1.00

chylphosphate + UDP-GlcNAc → dolichylpyrophosphoryl-*N*-acetylglucosamine [Dol-PP-GlcNAc] + UMP) in the sequence of the glycoside derivatives of dolichylpyrophosphate are latent. These reactions become activated in the presence of GTP if the membranes have been stripped of the bound ribosomes before incubation, leading to synthesis of dolichylpyrophosphate-linked oligosaccharides (Dol-PP-oligosaccharides) and to glycosylation of proteins. Although synthesis of dolichylpyrophosphoryl-chitobiose (Dol-PP-$GlcNAc_2$) is apparently the earliest effect of GTP (16), we recently obtained evidence that, as suspected before (17), the first reaction must also be included in the GTP-induced activities (15). In the presence of GTP, stripped rough microsomes completely assemble oligosaccharides of the type $Man_{\simeq 9}GlcNAc_2$ and transfer these oligosaccharides to protein (15, 17, 18).

The mechanism responsible for the stimulation of glycosylation activities by GTP is still unknown. It must be sought in the properties of the microsomal membrane rather than merely in the kinetics of an enzyme involved in the pathway of lipid intermediates. Indeed, we found subsequently that GTP also causes stripped rough microsomes to fuse and to form very large membrane-bounded vesicles (32), and that the experimental conditions which lead to membrane fusion and to active protein glycosylation in rough microsomes are identical. Hence, GTP alters the behavior of proteins and lipids within the stripped membranes. This alteration could result in the stimulated glycosylation activity, for the enzymes and the reagents involved, except for sugar nucleotide precursors, are integral constituents of the membranes.

To elucidate how GTP enhances glycosylation reactions in rough microsomes, we attempted to examine the transmembrane topology of the glycoside groups of the lipid and protein derivatives formed, using glycosidases or other enzymes as putative nonpenetrating probes. A prerequisite was that the membrane barrier be preserved after incubation with the sugar nucleotide precursors. Monitoring integrity of the membrane by the latency of mannose-6-phosphatase (2) and by the binding of esterase and nucleoside diphosphatase to microsomes (see reference 4), we found that the diffusion barrier of the membrane vanishes in stripped rough microsomes incubated with GTP under the precise conditions that lead to Dol-PP-oligosaccharide synthesis and to transfer of the synthesized oligosaccharides to proteins. This finding provides a reasonable explanation for the dependence of glycosylation activity in rough microsomes on both the removal of ribosomes and the presence of GTP in the reaction medium, and opens a new perspective over the transmembrane organization of protein core-glycosylation in the rough ER. We discuss these developments in this paper, even though we do not know yet which function of the rough ER depends on GTP in the intact cell. Some results have already been reported in abstract form (7, 42).

MATERIALS AND METHODS

Preparation and Treatment of Microsomal Subfractions: We prepared microsomes from female rat liver by differential centrifugation as described earlier (1), except that the microsomal pellet was not washed. They were brought to density equilibrium in a linear gradient of sucrose under the conditions used in previous studies from this laboratory (6). These conditions reduce hydrostatic pressure to a minimum (3). In some experiments (see Figs. 5 and 6) the gradient was divided into several subfractions of different density. In the other experiments, a single subfraction which contained the heavy material (density >1.2) was recovered. We designated this fraction heavy or rough microsomes; it consisted essentially of rough membrane-bounded vesicles (18) and was identical to the preparations used in our previous studies on the effects of GTP (15–18, 32).

Before incubation with GTP, we washed the microsome subfractions obtained from 10 g of liver three times by centrifugation for 30 min at 39,000 rpm in the No. 40 rotor (Beckman Instruments Inc., Palo Alto, CA) and resuspension in a solution of 0.25 M sucrose and 3 mM imidazole adjusted at pH 7.4 with HCl. The volume of solution was 50 ml for the washings, and 10 ml for the final resuspension (≃10 mg protein/ml). Alternatively, the sucrose solution used in the first two washings was supplemented with 3 mM EDTA. These preparations are designated washed (control) and EDTA-treated, respectively. When heavy microsomes are treated with EDTA, we speak of stripped rough microsomes because, on an RNA basis, ≃70% of the membrane-bound ribosomes are detached from the vesicles by EDTA (see reference 42 and Table II). In some experiments other treatments that strip the rough membranes were used. They are described in the experimental protocols (see Tables II and III, and Fig. 6).

Incubation of Microsomal Subfractions with Nucleotides: Incubation with GTP or with other nucleotides was carried out at 37°C, in a medium that contained 40 mM Tris-HCl buffer, pH 7.4, 30 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 2.5 mM dithiothreitol, 1 mM ATP, 10 mM phosphoenolpyruvate and 50 μg pyruvate kinase (200 U/mg) per milliliter (medium A). The concentration of other constituents present is given in the experimental protocols. After incubation, the mixture was immediately assayed for free and total activity of mannose-6-phosphatase, or ice-cooled and centrifuged for 30 min at 29,000 rpm in the No. 30 Beckman rotor equipped with minitubes (5 × 42 mm). Esterase or nucleoside diphosphatase was then assayed in the supernate (soluble activity) and in the pellet after resuspension in 0.25 M sucrose buffered at pH 7.4 by 3 mM imidazole-HCl (bound activity).

To incorporate *N*-acetylglucosamine and mannose into endogenous lipid and protein, microsome subfractions were incubated at 37°C in medium A supplemented with ^{14}C-labeled sugar nucleotide precursors at the concentrations given in the legends of tables and figures. Reactions were ended by addition of 10 vol of 10% ice-cold trichloroacetic acid (TCA), or of 10 vol of chloroform/methanol (3/2; vol/vol) and 1.6 vol of 4 mM $MgCl_2$.

Biochemical Determinations: Mannose-6-phosphatase activity was assayed by incubation at 30°C in 0.55 ml of medium at pH 6.5, which contained 0.25 M sucrose, 20 mM mannose-6-phosphate, 2.5 mM EDTA, and 20 mM histidine. Blanks were run in the absence of mannose-6-phosphate. After 10 min, proteins were precipitated by addition of 2 ml of 10% ice-cold TCA, and phosphate was determined by the method of Fiske and Subbarow (13). The result is referred to as free or total activity, depending on whether the microsome subfraction (0.1 ml) was diluted by addition of 0.5 vol of 0.25 M sucrose, or Triton X-100 (2 mg/ml in 0.25 M sucrose), immediately before the enzyme reaction.

Labeled sugars incorporated into lipid and protein derivatives were determined according to the analytical methods used previously (16–18). Radioactivity was counted by liquid scintillation in (*a*) the TCA-insoluble material after mild acid hydrolysis, which gives incorporation into glycosylated protein, (*b*) the material released by mild acid hydrolysis from washed TCA precipitates, which gives incorporation into glycosylated derivatives of dolichylpyrophosphate, and (*c*) the radioactive spots revealed by autoradiography after thin-layer chromatography of the chloroform/methanol extract, which give the amounts of label in Dol-PP-GlcNAc and Dol-PP-$GlcNAc_2$.

Protein (29), RNA (14), phospholipid, esterase, nucleoside diphosphatase, NADH cytochrome *c* reductase, glucose-6-phosphatase, 5′-nucleotidase (5), protein-*N*-acetylglucosaminyltransferase (43), and *N*-acetylglucosamine-phosphotransferase (34) were assayed according to the methods described in the quoted articles. All enzyme activities are expressed in standard units, i.e. micromoles of substrate transformed per minute.

Materials: Mannose-6-phosphate (grade 1), Triton X-100, bovine α-lactalbumin, puromycin, and ethyleneglycol-*bis*-(β-aminoethyl ether)*N,N′*-tetraacetate (EGTA) were obtained from the Sigma Chemical Co. (St. Louis, MO). UDP-[^{14}C]GlcNAc (300 Ci/mol) and GDP-[^{14}C]Man (200 Ci/mol) were obtained from the Radiochemical Centre (Amersham, UK). EDTA was purchased from E. Merck A.G. (Darmstadt, Federal Republic of Germany [FRG]), and tunicamycin from Calbiochem-Behring Co. (La Jolla, CA). dGTP and guanylyl(β,γ-methylene)-diphosphonate (GMP-PCP) were obtained from Boehringer Mannheim GmbH (Mannheim, FRG). Other reagents were those used previously (5, 18).

RESULTS

Structure-linked Latency of Mannose-6-Phosphatase

The latency of glucose-6-phosphatase assayed with mannose-6-phosphate, a substrate that is not transported by the

glucose-6-phosphate carrier, is an index of the permeability barrier of microsomal membranes derived from the ER (2). In Fig. 1, we show the changes in free and total activity of mannose-6-phosphatase which occurred upon incubation of rough microsomes at 37°C under various conditions.

Before incubation ≃90% of the enzyme activity was latent, indicating a low permeability to mannose-6-phosphate in both the rough and the stripped membranes. GTP had no perceptible effect on the free and total activities. After incubation for 120 min, a slight loss (≃25%) of total activity was apparent under all the conditions used, and the free activity was only moderately enhanced in control rough microsomes incubated with or without GTP, and in stripped rough microsomes incubated in the absence of GTP. The latency levels still ranged between 75 and 80%.

Stripped rough microsomes incubated with GTP behaved quite differently. After 30-min incubation, the free activity almost accounted for the total activity, the latency being reduced to only 14%. This value is confirmed by the results at 120 min, and suggests a considerable increase of the membrane permeability to mannose-6-phosphate.

The disappearance of the structure-linked latency of mannose-6-phosphatase in stripped rough microsomes incubated with GTP is time- and temperature-dependent. In the experiment reported in Fig. 2, stripped rough microsomes were incubated for 120 min at various temperatures, or for various times at 37°C. A significant decrease of the latency occurred at 15°C, or above, in the presence of GTP. The phenomenon was maximum after ≃30 min at 37°C.

The sensitivity of stripped rough microsomes to GTP is high. Indeed, when the concentration of this nucleotide was varied the maximum free activity of mannose-6-phosphatase was reached with ≃30 μM GTP, and the half-maximum effect was produced by ≃10 μM GTP (Fig. 3). In previous studies we have shown that 50 μM GTP is sufficient for maximum stimulation of Dol-PP-oligosaccharide synthesis (18).

Release of Nucleoside Diphosphatase and Esterase by GTP

The easy permeation of mannose-6-phosphate into stripped rough microsomes after incubation with GTP may reflect a gross alteration of the membrane barrier, or a more selective

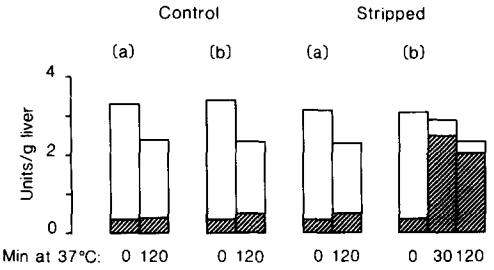

FIGURE 1 Increase of the free mannose-6-phosphatase activity in stripped rough microsomes incubated with GTP. Control rough microsomes and stripped rough microsomes obtained from the same preparation were incubated in medium A (see Materials and Methods) for various times given under each graph. The incubation medium contained the microsomes derived from 0.4 g liver/ml and was supplemented with 0.25 M sucrose (a), or with 0.25 M sucrose and 0.5 mM GTP (b). The total and free activities of mannose-6-phosphatase were determined afterwards. The free activities are shown by the hatched sections of bars; the upper open sections give the latent activities (excess of total over free activities).

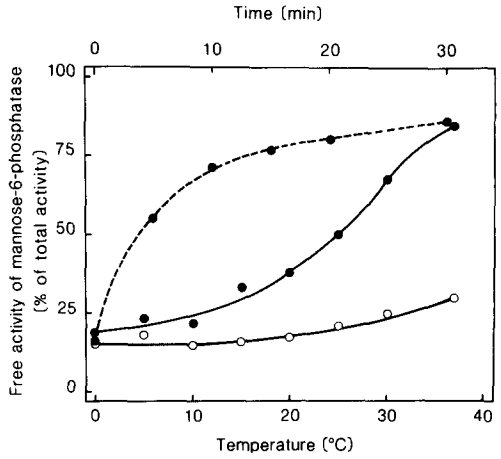

FIGURE 2 Time course and temperature dependence of the increase in free mannose-6-phosphatase activity. Stripped rough microsomes were incubated in medium A (see Materials and Methods) supplemented with 0.25 M sucrose (○), or 0.25 M sucrose and 0.5 mM GTP (●). The incubation medium contained the microsomes derived from 0.4 g liver/ml. Free and total activities of mannose-6-phosphatase were assayed after incubation for 120 min at various temperatures (solid lines), or for various times at 37°C (dashed line).

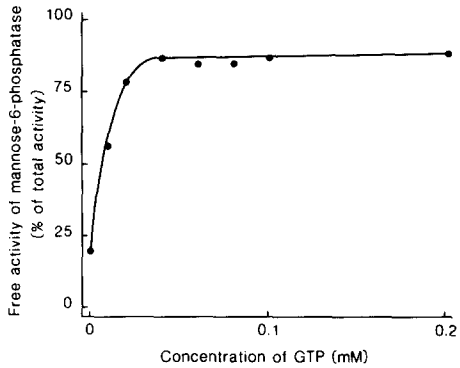

FIGURE 3 Dependence of the increase in free mannose-6-phosphatase activity on concentration of GTP. Stripped rough microsomes were incubated in medium A (see Materials and Methods) supplemented with 0.25 M sucrose and GTP at various concentrations. The incubation medium contained the microsomes derived from 0.4 g liver/ml. After 30 min, free and total activities of mannose-6-phosphatase were assayed.

modification allowing the intake of a restricted number of metabolites. To better characterize the membrane effects of GTP, we examined the centrifugation behavior of nucleoside diphosphatase and esterase in rough microsomes incubated under the various conditions used in Fig. 1. These enzymes are located in the lumen, or weakly bound to the luminal surface of the ER (for a review, see 4), and thus sediment with microsomes unless the membrane be markedly altered.

Esterase and nucleoside diphosphatase were released in the medium concomitant with the disappearance of the latency of mannose-6-phosphatase (Fig. 4). Indeed, whereas these enzymes were largely recovered in the sediment after incubation of control rough microsomes with or without GTP, and of stripped rough microsomes without GTP, a significant fraction was found in the supernatant fluid when the stripped preparation was incubated for 30 or 120 min in the presence of 0.5 mM GTP. For esterase the total activity was constant, and ~50% of this enzyme was soluble in GTP-treated stripped preparations. For nucleoside diphosphatase the final

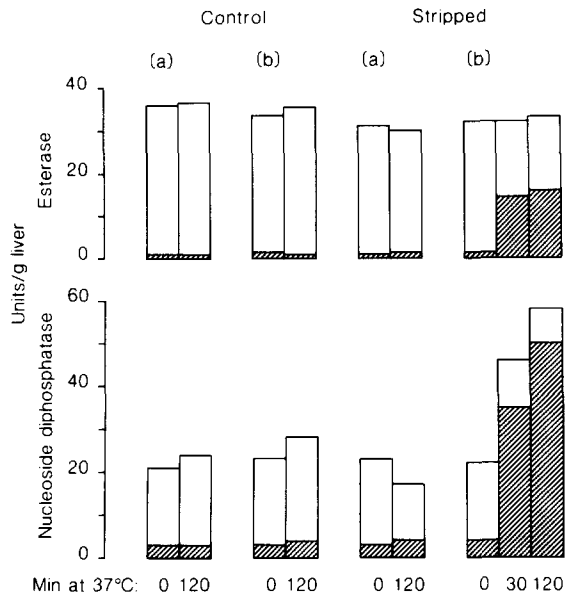

FIGURE 4 Release of esterase and nucleoside diphosphatase upon incubation with GTP. Control rough microsomes and stripped rough microsomes obtained from the same preparation were incubated as described in Fig. 1 without GTP (*a*), or in the presence of 0.5 mM GTP (*b*), and centrifuged after the times given under each graph (see Materials and Methods). Esterase and nucleoside diphosphatase were assayed in the resulting pellets (open sections of the bars) and supernates (closed sections of the bars).

level of soluble activity was 85% but the total activity was increased 2.0- to 2.6-fold. Activation of nucleoside diphosphatase following solubilization and removal of the membranes is in keeping with the presence of an inhibitor of the enzyme in the membranes previously reported by others (27). In spite of this interference, it is seen that the activity recovered in the sediment is reduced to ≃40% of the original value in the stripped preparation incubated for 30 min with GTP.

Effect of Other Nucleotides

GTP is the only nucleotide known to cause fusion of stripped rough vesicles (32) and to allow them to core-glycosylate proteins through the pathway of lipid intermediates (17). Therefore, we compared the effect of a number of nucleotides on the latency of mannose-6-phosphatase and on the association of esterase with microsomal vesicles (Table I).

It is implicit in the results presented so far (Figs. 1–4) that GTP cannot be substituted by ATP, which was regularly present in the reaction medium at 1 mM concentration (see Materials and Methods). It cannot be substituted either by other major nucleoside triphosphates, including dGTP, or by the nonhydrolyzable analogue GMP-PCP (Table I). The specificity with respect to GTP is particularly apparent, as the other nucleotides did not act at a concentration ≃20-fold that at which GTP gives a maximal effect (Fig. 3).

Dependence of GTP-induced Alteration of the Membrane Barrier and of the Glycosylation Activity on the Removal of Ribosomes

The effect of GTP on the latency of mannose-6-phosphatase has been investigated using rough microsomes previously treated with various reagents in order to know whether the sensitivity of the membrane to GTP results from the removal of ribosomes, or from another effect of EDTA. In the experiment shown in Table II, ribosomes were detached from heavy microsomes by treatment with EDTA, sodium pyrophosphate, KCl, or puromycin in the presence of KCl and $MgCl_2$. The extent of ribosome removal is estimated by the decrease of the RNA content. On subsequent incubation the free activity of mannose-6-phosphatase increased when GTP was added to the medium. Both the release of RNA and the GTP-induced alteration of the membranes occurred, but were less after treatment with puromycin. In contrast, GTP had no effect on heavy microsomes treated with KCl and $MgCl_2$, which still bore 76% of the RNA present in the control. We reported earlier (32) that GTP induced fusion of vesicles, synthesis of Dol-PP-oligosaccharides and protein glycosylation after treatment of heavy microsomes under the precise conditions shown here to increase the free activity of man-

TABLE I

Effect of Various Nucleotides on the Levels of Free Mannose-6-Phosphatase and Soluble Esterase

Nucleotide added	Free mannose-6-phosphatase	Soluble esterase
(*0.5 mM*)	(*% of total activity*)	
—	23	2
GTP	74	32
UTP	25	2
ITP	31	2
CTP	21	2
GMP-PCP	29	1
dGTP	30	2

Stripped rough microsomes (0.4 g liver/ml) were incubated in Medium A, which included 1 mM ATP (see Materials and Methods) and was supplemented with various nucleotides as indicated. After 45 min, the preparations were assayed for free and total activity of mannose-6-phosphatase, while a portion was centrifuged to determine the fraction of esterase in soluble form. Total activities were 1.64 ± 0.09 and 30.9 ± 2.2 U/g liver (means ± SD) for mannose-6-phosphatase and esterase, respectively.

TABLE II

Effect of GTP on the Latency of Mannose-6-Phosphatase in Rough Microsomes Subjected Previously to Various Treatments

Treatment of heavy microsomes	RNA	Free mannose-6-phosphatase	
		a	b
	(*% of control*)	(*% of total activity*)	
Control	100	22.5	25.3
EDTA	32	24.5	91.0
PP_i	35	20.7	68
KCl	18	24.5	80.7
KCl + $MgCl_2$	76	25.5	22.5
Puromycin	58	26.1	42.0

Heavy microsomes have been washed with sucrose (Control) or treated with EDTA as described under Materials and Methods. Other portions of the heavy microsome subfraction have been similarly treated twice with sucrose solutions which contained 5 mM sodium pyrophosphate pH 7.4 (PP_i), or 0.75 M KCl, or 0.75 M KCl and 5 mM $MgCl_2$. Another portion (Puromycin) was treated with a sucrose solution that contained 0.75 M KCl, 5 mM $MgCl_2$, and 1 mM puromycin, kept at 0°C for 30 min before centrifugation, and washed in the KCl + $MgCl_2$ medium. All preparations were finally washed and resuspended in 0.25 M sucrose as in the usual protocol. RNA amounted to 1.8 mg/g liver in the control, and was reduced after the various treatments as indicated by the percent values. The preparations were incubated for 45 min in medium A which contained the material derived from 0.4 g liver/ml and was supplemented with 0.25 M sucrose (*a*) or 0.25 M sucrose and 0.5 mM GTP (*b*). The incubated preparations were then assayed for free and total activity of mannose-6-phosphatase. Total activities were not changed by the treatments or the presence of GTP, and amounted to 2.17 ± 0.08 U/g liver (mean ± SD).

TABLE III

Effect of GTP on the Latency of Mannose-6-Phosphatase and the Incorporation of N-Acetylglucosamine into Lipid and Protein by Rough Microsomes Treated with Chelating Agents at Various Concentrations

Treatment of heavy microsomes	RNA	Free activity of mannose-6-phosphatase	Incorporation of *N*-acetylglucosamine		
			Dol-PP-GlcNAc	Dol-PP-GlcNAc$_2$	Protein
	(*% of control*)	(*% of total activity*)	(*pmol sugar/mg phospholipid*)		
(a) Undiscriminated	—	(24.1 ± 2.0)	(138 ± 13)	(29.7 ± 1.5)	(5.7 ± 0.7)
(b) Control	100	24	146	77	7
0.25 mM EDTA	81	35	171	122	13
0.50 mM EDTA	62	50	147	244	33
1 mM EDTA	40	88	71	549	151
5 mM EDTA	33	96	58	497	158
10 mM EGTA	86	26	133	108	11
50 mM EGTA	41	97	54	514	114

Heavy microsomes have been prepared and treated as described in the Materials and Methods section, except that EDTA was used at several concentrations, or substituted by EGTA. RNA amounted to 1.87 mg/g liver in the control and was lowered by the chelating agents as shown by the percent values. The preparations were incubated for 60 min in medium A which contained the material derived from 0.4 g liver/ml and was supplemented with 0.25 M sucrose (*a*), or 0.25 M sucrose and 0.5 mM GTP (*b*). Free and total activities of mannose-6-phosphatase were determined afterwards. Total activities were independent of the conditions used and amounted to 3.23 ± 0.16 U/g liver (mean ± SD). The preparations were also incubated as described above in the presence of 6 μM UDP-[^{14}C]GlcNAc, to establish the incorporation of *N*-acetylglucosamine into Dol-PP-GlcNAc, Dol-PP-GlcNAc$_2$, and protein. In the absence of GTP free activities of mannose-6-phosphatase and *N*-acetylglucosamine incorporation into lipid and protein did not show any significant variation after the treatment; they are undiscriminatingly given within brackets as means ± SD. The other values of mannose-6-phosphatase activity and sugar incorporation were obtained in the presence of GTP.

nose-6-phosphatase. Clearly, the three effects of GTP, including the change in permeability, are similarly conditioned by the removal of ribosomes.

The relationship between the removal of ribosomes and the sensitivity of rough membranes to GTP has been further investigated on preparations stripped gradually by treatment with EDTA or EGTA at various concentrations (Table III). The RNA content of washed rough microsomes (control) is ~80% of the original content in the unwashed heavy microsomes (18). The maximal effect of GTP on latency of mannose-6-phosphatase, synthesis of Dol-PP-GlcNAc$_2$ and protein glycosylation requires a drop of the RNA load to 40% of the control (1 mM EDTA, 50 mM EGTA). A significant change in latency of mannose-6-phosphatase and in synthesis of Dol-PP-GlcNAc$_2$ in the presence of GTP starts at ≃80% of the RNA load in the control (≃65% of the load in unwashed heavy microsomes).

Response of Distinct Microsome Subfractions

Because rough microsomes become permeable on incubation with GTP only after release of RNA, it was of interest to know whether smooth microsomes are sensitive to GTP without any treatment, become sensitive after treatment with EDTA, or remain insensitive even after this treatment. To discriminate between these possibilities the response of the ER membranes to GTP in various microsome subfractions has been compared (Fig. 5). These subfractions were prepared by density equilibrium centrifugation in a sucrose gradient; they differed from one another by the average ribosome load of the vesicles as illustrated by the distributions of RNA and glucose-6-phosphatase. All subfractions were treated with EDTA before they were challenged by incubation with GTP.

Fig. 5 shows the distribution of free and total mannose-6-phosphatase, and of soluble and bound esterase, determined after challenging. As expected, total mannose-6-phosphatase and esterase were distributed the same as glucose-6-phosphatase. In contrast, the distribution of the free mannose-6-phosphatase activity was rather similar to that of RNA. The latency of the enzyme was high (≃92%) in the two light subfractions, undetectable in the two dense subfractions, and

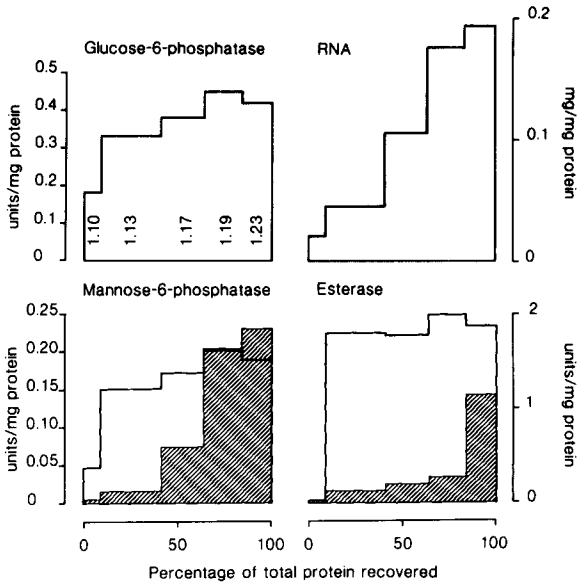

FIGURE 5 Effect of GTP on the latency of mannose-6-phosphatase and on the release of esterase in microsomal subfractions of different density. Microsomes were divided into five subfractions of different density by equilibrium centrifugation in a linear gradient of sucrose. A portion of each subfraction was saved for determination of protein, RNA, and glucose-6-phosphatase activity. The remainder was treated with EDTA, incubated for 45 min in medium A (see Materials and Methods), supplemented with 0.25 M sucrose and 0.5 mM GTP, and finally assayed for free and total activity of mannose-6-phosphatase and for soluble and bound activity of esterase. Fractions are plotted from left to right in the order of increasing density. Average density values are indicated on the plot of glucose-6-phosphatase. Distributions give the specific activities of enzymes and the specific content in RNA, versus the content in protein accumulated from the top to the bottom of the gradient (100% = 42 mg protein/g liver). A shaded profile corresponding to free mannose-6-phosphatase, or soluble esterase, is superimposed on the distribution of the total activities.

57% in the intermediate subfraction. Esterase became markedly released only in the densest subfraction.

Activation of the lipid-linked glycosylation pathway by GTP after previous treatment with EDTA or pyrophosphate

was also restricted to rough microsomes. This characteristic is illustrated by the results presented in Fig. 6. Microsome subfractions of different equilibrium density were assayed for the ER constituents RNA and glucose-6-phosphatase, and for protein-*N*-acetylglucosaminyltransferase and 5′-nucleotidase which are markers of Golgi and plasma membrane elements, respectively (43). All subfractions were either washed with sucrose, or treated with sodium pyrophosphate under conditions that detach the ribosomes from the rough membranes, and then incubated with labeled UDP-GlcNAc in the presence of GTP to establish the incorporation of *N*-acetylglucosamine into the endogenous lipid and protein. The treatment with pyrophosphate altered the glycosylation patterns in the rough microsome subfractions exclusively. The synthesis of Dol-PP-$GlcNAc_2$ is seen only in dense subfractions treated with pyrophosphate. Labeled *N*-acetylglucosamine was incorporated into protein in smooth and in rough microsomes. However, protein glycosylation in smooth microsomes was not influenced by the preliminary treatment with pyrophosphate, contrary to protein glycosylation in rough microsomes. The distribution pattern of glycosylated protein in the subfractions washed with sucrose is similar to that of protein-*N*-acetylglucosaminyltransferase and largely reflects the transfer of peripheral *N*-acetylglucosamine residues to preexisting glycoproteins in microsomes derived from the Golgi complex. In contrast, incorporation of labeled *N*-acetylglucosamine into protein in the dense subfractions consists of lipid-mediated core glycosylation of protein, as demonstrated elsewhere (15). In other experiments the subfractions were treated with pyrophosphate, or EDTA, and incubated with the labeled sugar nucleotide precursor in the absence of GTP. The glycosylation patterns were similar to those presented as hatched histograms in Fig. 6 (results not shown).

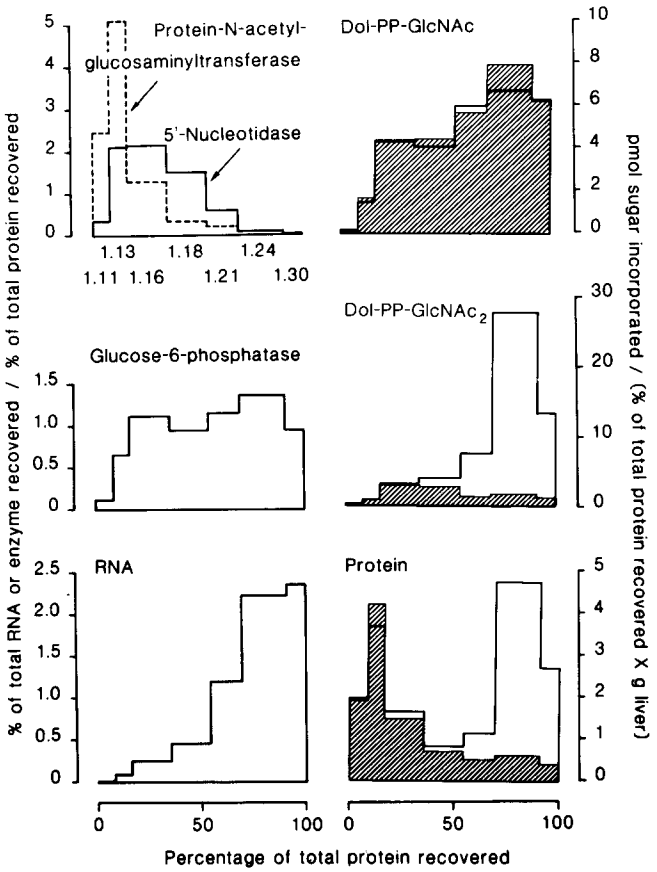

FIGURE 6 Effect of pyrophosphate treatment on glycosylation activity in microsomal subfractions of different density. Microsomes were divided into seven subfractions of different density as in the experiment of Fig. 5. The distributions of protein-*N*-acetylglucosaminyltransferase, 5′-nucleotidase, glucose-6-phosphatase, and RNA are presented on the left side in percentage values relative to the total microsomes. Average densities of fractions are given under the upper histograms. Each subfraction was divided into two portions, one washed with sucrose (*control*), the other treated with pyrophosphate (PP_i), exactly as described in Table II. The *control* and PP_i portions derived from 0.04–0.1 g liver (≃0.15 mg phospholipid) were incubated for 60 min in 0.4 ml medium A (see Materials and Methods), supplemented with 0.125 M sucrose, 0.5 mM GTP, and 3.2 μM UDP-[^{14}C]GlcNAc. Amounts of the labeled precursor incorporated into Dol-PP-GlcNAc, Dol-PP-$GlcNAc_2$, and protein are shown on the right side in absolute values. Incorporation patterns obtained in the *control* (shaded) and in the PP_i portions are superimposed.

In conclusion, both permeability of the vesicular membranes and protein glycosylation via the pathway of lipid intermediates are inducible by GTP only in the rough microsomes. We ruled out a number of trivial explanations of this specific character of rough microsomes in experiments designed to verify that sugar nucleotide precursors and GTP do not rapidly disappear from the reaction medium in low density subfractions. The absence of Dol-PP-$GlcNAc_2$ synthesis and lipid-mediated glycosylation of protein in smooth microsomal membranes could result from the lack of some enzymes, particularly of the glycosyltransferase converting Dol-PP-GlcNAc into Dol-PP-$GlcNAc_2$. Although we cannot rule out this possibility, we must in any way conclude that GTP specifically acts on the rough membranes because its effects on the permeability are confined to the rough microsome fractions. Our conclusion is strengthened by the dependence of this action on the detachment of ribosomes.

Latency of Dol-PP-GlcNAc and Dol-PP-$GlcNAc_2$ Synthesis in Rough Microsomes

The close correlation between the glycosylation activity and the membrane permeability induced by GTP in stripped rough microsomes suggests that the membrane barrier might be responsible for the low glycosylation activity in sealed vesicles. In consideration of this possibility the influence of various detergents on the glycosylation reactions involving the microsomal dolichylphosphate has been examined.

Influence of Triton X-100 on the level of microsome-bound esterase and on the incorporation of labeled *N*-acetylglucosamine into glycoside derivatives of dolichylpyrophosphate is shown in Fig. 7. The plot of soluble esterase versus the concentration of Triton X-100 is roughly sigmoidal and shows that the bulk of the enzyme (≃80%) is released in the presence of 0.1% detergent in the medium used. In the absence of Triton X-100, a significant amount of Dol-PP-GlcNAc was formed, whereas the level of Dol-PP-$GlcNAc_2$ was much lower. Raising the concentration of Triton X-100 progressively inhibited synthesis of Dol-PP-GlcNAc and of Dol-PP-$GlcNAc_2$ till the concentration of 0.1%. This inhibition phase was followed by a steep rise in the level of Dol-PP-$GlcNAc_2$, which became maximal at 0.2–0.3% Triton X-100. The amount of Dol-PP-GlcNAc also rose within this concentration range, without reaching the original level, most likely because it was converted into Dol-PP-$GlcNAc_2$. In our mem-

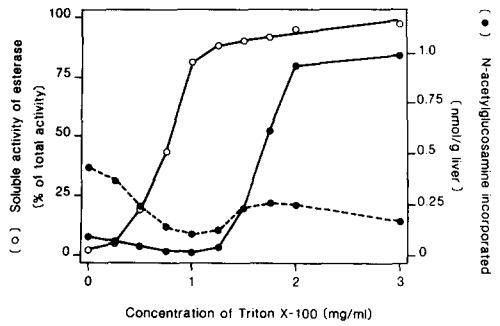

FIGURE 7 Effect of Triton X-100 on the binding of esterase and the synthesis of Dol-PP-GlcNAc$_2$ from endogenous dolichylphosphate. Stripped rough microsomes were incubated in medium A (see Materials and Methods), which contained the material derived from 0.4 g liver (≈0.92 mg phospholipid/ml) and was supplemented with 0.25 M sucrose and Triton X-100 at the concentrations given in abscissa. After 60 min, they were centrifuged to determine the distribution of esterase (○) between the pellets and supernates. Another preparation was incubated under identical conditions, except that the medium contained 3.3 μM UDP-[^{14}C]GlcNAc. After 60 min lipids were extracted in chloroform/methanol and separated by thin-layer chromatography to determine incorporation of labeled *N*-acetylglucosamine into Dol-PP-GlcNAc (●---●) and Dol-PP-GlcNAc$_2$ (●——●).

brane preparations the diglycoside derivative is not formed from preexistent Dol-PP-GlcNAc, but by sequential transfer of *N*-acetylglucosamine-1-phosphate and *N*-acetylglucosamine from labeled UDP-GlcNAc to dolichylphosphate (15). From the data of Fig. 7, it can be computed that the total amount of dolichol involved in labeled Dol-PP-GlcNAc and Dol-PP-GlcNAc$_2$ was 50% greater in the presence of 0.2% Triton X-100 than without detergent (90 and 60 pmol/g liver, respectively). Thus, in sealed vesicles only a part of dolichylphosphate reacts with UDP-GlcNAc.

A similar experiment was carried out in the presence of GDP-Man, with and without GTP (Fig. 8). The GTP-induced *N*-acetylglucosamine incorporation into glycoside derivatives of dolichylpyrophosphate was also markedly inhibited (up to 90%) in the 0–0.1% range of Triton X-100 concentration. At 0.2% the amount of label incorporated was nearly identical to that found in the presence of GTP without Triton X-100, but did not depend on GTP anymore. Analysis of lipids by thin-layer chromatography showed a wide spectrum of labeled glycoside derivatives after incubation with GTP in the absence of Triton X-100, whereas only mono-, di-, and trisaccharide derivatives were revealed after incubation in the presence of 0.2% Triton X-100 (not shown).

The latency of the reaction converting Dol-PP-GlcNAc into Dol-PP-GlcNAc$_2$ is also apparent in the experiment reported in Fig. 9. The monoglycoside derivative was formed during a first incubation with UDP-[^{14}C]GlcNAc in the absence of Triton X-100. Afterwards tunicamycin was added to inhibit any new synthesis of Dol-PP-GlcNAc and the reaction was continued in the presence of various concentrations of Triton X-100. The monoglycoside derivative was almost quantitatively transformed into the diglycoside in the presence of 0.2% or more Triton X-100.

Thus, regarding the synthesis of Dol-PP-GlcNAc and Dol-PP-GlcNAc$_2$, 0.2% Triton X-100 and GTP have strikingly similar effects. However, under our experimental conditions the detergent cannot lead to synthesis of Dol-PP-oligosaccharides (see above) and to lipid-mediated glycosylation of protein (Table IV). The statistics relative to sugar incorporation in the absence of GTP (Table IV, *a*) derive from results obtained at the various concentrations of the detergent and demonstrate that it was without noticeable effect on protein glycosylation. As expected (17, 18), incorporation of *N*-acetylglucosamine and mannose into protein was enhanced by GTP (compare in Table IV values *a* with values *b* without Triton X-100). The detergent strongly inhibited the GTP-induced glycosylation of protein even at 0.025%, a concentration still below that needed for release of esterase (Fig. 7).

Other detergents, including sodium deoxycholate, digitonin, Emulgen 913, lysophosphatidylcholine, and octylglucoside, were used in experiments similar to that shown in Fig. 7. In general, they inhibited Dol-PP-GlcNAc synthesis as does

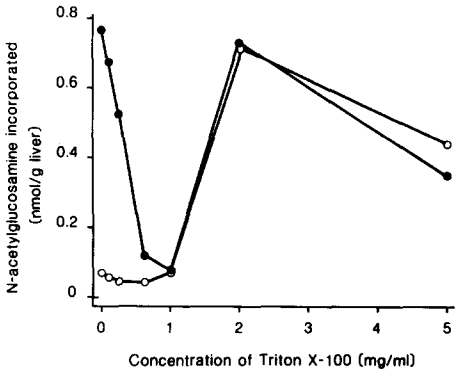

FIGURE 8 Inhibition by Triton X-100 of the GTP-induced synthesis of glycosylated derivatives of dolichylpyrophosphate. Stripped rough microsomes (0.4 g liver/ml) were incubated for 60 min in medium A (see Materials and Methods) with 3.3 μM UDP-[^{14}C]-GlcNAc plus 5.8 μM GDP-Man, without GTP (○) or in the presence of 0.5 mM GTP (●). The medium also contained 0.1 M sucrose and various amounts of Triton X-100. Label incorporation into dolichylpyrophosphate derivatives was measured on the material released from TCA precipitates by mild acid hydrolysis. Identical results were obtained when glycosylated lipids were extracted in chloroform/methanol/water (10/10/3, vol/vol/vol).

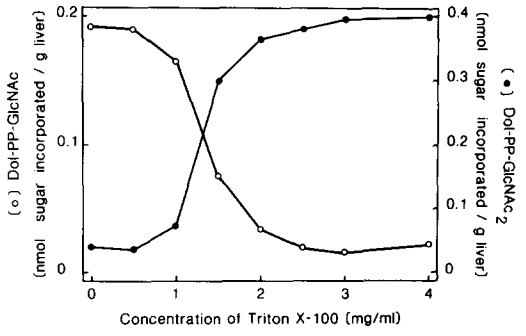

FIGURE 9 Effect of Triton X-100 on the transformation of Dol-PP-GlcNAc into Dol-PP-GlcNAc$_2$. Stripped rough microsomes were incubated in medium A (see Materials and Methods) which contained the material derived from 0.4 g liver/ml and was supplemented with 0.25 M sucrose and 6 μM UDP-[^{14}C]GlcNAc. After 30 min, 2.5 μg/ml tunicamycin was added and incubation was continued for 10 min. Triton X-100 was then added to reach the concentration given in abscissa and the incubation was continued for 60 min. Lipids were then extracted in chloroform/methanol and separated by thin-layer chromatography to determine the amounts of labeled *N*-acetylglucosamine in Dol-PP-GlcNAc (○) and Dol-PP-GlcNAc$_2$ (●).

Triton X-100 in the 0–0.1% range, and never led to high levels of Dol-PP-GlcNAc$_2$.

Bulk Biochemical Properties of Stripped Rough ER Membranes Incubated with GTP

Attempts to identify a biochemical modification underlying the GTP-induced permeability of stripped rough microsomes have been unsuccessful so far. Stripped rough microsomes incubated with or without GTP gave identical peptide patterns after SDS-PAGE and identical lipid patterns after thin-layer chromatography of chloroform/methanol extracts.

In fact, incubation with GTP appears to be a most gentle means to increase the permeability of membranes derived from the rough ER. This is illustrated by the data reported in Table V. GTP renders stripped rough microsomal membranes permeable without recognizable change in their biochemical features, in contrast to Triton X-100, which at the concentration required for synthesis of Dol-PP-GlcNAc$_2$ removes part of the phospholipid and of the integral membrane proteins (e.g., *N*-acetylglucosaminephosphotransferase, and NADH cytochrome *c* reductase).

TABLE IV

Inhibition of the GTP-induced Glycosylation of Protein by Triton X-100

	Labeled sugar incorporated	
Triton X-100	*N*-Acetylglucosamine	Mannose
(mg/ml)	(pmol sugar/mg phospholipid)	
(a) Undiscriminated	(4.4 ± 1.7)	(94 ± 12)
(b) 0	683	2,490
0.1	552	1,880
0.25	179	514
0.50	27	151
1.0	2	84
2	2	66
5	2	64

Stripped rough microsomes (0.4 g liver/ml) were incubated for 60 min in medium A (see Materials and Methods) with 3.3 μM UDP-[^{14}C]GlcNAc plus 6.4 μM GDP-Man, or 3.5 μM UDP-GlcNAc plus 6.4 μM GDP-[^{14}C]Man, without GTP (a) or in the presence of 0.5 mM GTP (*b*). The medium contained, in addition, 0.1 M sucrose, 2.4 mg/ml bovine α-lactalbumin, and various amounts of Triton X-100. Label incorporation into protein was measured on the residues after extraction with chloroform/methanol (3/2, vol/vol) and chloroform/methanol/water (10/10/3, vol/vol/vol). In the absence of GTP incorporation of *N*-acetylglucosamine and mannose did not significantly vary according to the concentration of detergent; the values are undiscriminatingly given within brackets as means ± SD. The other incorporation values were obtained in the presence of GTP at the specified concentrations of Triton X-100.

DISCUSSION

Our findings provide additional information on the changes which take place in stripped membranes of the rough microsomes on incubation with GTP. The increased activity of the dolichol-dependent protein glycosylation originally discovered (16–18), the membrane fusion phenomenon leading to very large membrane-bounded vesicles observed later (32), and the greater permeability of the vesicular membranes reported here are three closely related events. All of them depend on GTP in a specific manner (Table I) conditioned by the removal of ribosomes before incubation with this nucleotide (Tables II and III) and are induced only in rough microsomes (Figs. 5 and 6) (this has not been shown for fusion). They probably derive from a single primary action of GTP. The first question is to examine which relationships exist between the various resulting events.

Considering first the possible link of permeability with fusion, it is conceivable that leakiness be a transient property, coinciding with the fusion of vesicles. This would imply a molecular mechanism that does not keep the coalescing membranes tight throughout the fusion process. Although membrane fusion is currently thought to occur without leakage of the vesicular content, a transient permeability might reasonably explain how esterase and nucleoside diphosphatase partly escape from the vesicles. However, this explanation does not hold for the high level of free mannose-6-phosphatase activity revealed in our experiments. Indeed, most fusion events occur during the incubation with GTP, before addition of mannose-6-phosphate. Another inconsistency with permeability resulting from fusion events is that apparently a significant portion of the vesicles did not fuse after 120-min incubation (32), whereas the degree of latency was already reduced to 10–15% after 30 min. Thus, altering the permeability barrier is probably a more direct consequence of the primary action of GTP on the membrane.

The possible link between the GTP-induced permeability and glycosylation activity of microsomal vesicles is easier to envision. When rough microsomes from rat liver are supplied with UDP-GlcNAc and GDP-Man the reactions leading to synthesis of Dol-PP-oligosaccharides and to *N*-glycosylation of proteins are latent, but become active in stripped membranes after addition of GTP (15–18). From the structure-linked latency of mannose-6-phosphatase (Fig. 1) and the centrifugation behavior of esterase and nucleoside diphosphatase (Fig. 4) we see that the vesicles remain fairly sealed under the various conditions in which the glycosylation activity is latent, and that the membrane barrier is markedly altered in

TABLE V

Release of Various Membrane Constituents by Triton X-100 and GTP

	N-Acetylglucosamine-phosphotransferase		NADH cytochrome *c* reductase		Phospholipid	
Conditions	Pellet	Supernate	Pellet	Supernate	Pellet	Supernate
	(μU)		(U)		(mg)	
4°C	140 (152)	1	4.4 (5.3)	0.2	2.2 (2.9)	0.4
37°C	135	1	4.0	0.3	2.1	0.3
4°C + Triton X-100	60	83	3.2	2.0	1.2	1.6
37°C + GTP	141	1	4.7	0.2	2.7	0.5

Stripped rough microsomes (0.4 g liver/ml) were incubated for 30 min in medium A (see Materials and Methods) supplemented with 0.25 M sucrose, either at 37°C with 0.5 mM GTP or without GTP, or at 4°C with 2 mg Triton X-100/ml or without Triton X-100. The incubated material was centrifuged for 60 min at 29,000 rpm and various constituents were assayed in the pellets and in the supernates. Values in the total material after incubation at 4°C without Triton X-100 are given between brackets. All values refer to 1 g liver (≃10 mg protein in rough microsomes).

the unique situation in which the dolichol-mediated glycosylation of protein is active. This correlation suggests that one or several enzymes involved in Dol-PP-oligosaccharide synthesis act at the luminal aspect of the rough microsomes and cannot receive the added sugar nucleotide precursors unless the membrane barrier be overcome in some way. It is highly significant that the GTP-induced permeability and the membrane disaggregation by Triton X-100 lead partly to the same result, allowing to glycosylate a greater amount of endogenous dolichylphosphate and to convert Dol-PP-GlcNAc into Dol-PP-$GlcNAc_2$. This demonstrates that membrane barriers hinder early steps in the assembly of Dol-PP-oligosaccharides.

Permeation of UDP-GlcNAc into the vesicular lumen could be a prerequisite for Dol-PP-GlcNAc to be converted into Dol-PP-$GlcNAc_2$, since this reaction clearly depends on an alteration of the membrane by GTP or Triton X-100. The latency of *N*-acetylglucosaminephosphotransferase reaction is less obvious, for Dol-PP-GlcNAc is partly formed in unaltered rough microsomes. This does not completely rule out the possibility that the *N*-acetylglucosaminephosphotransferase reaction driving Dol-PP-oligosaccharide synthesis and core glycosylation of proteins in GTP-altered membranes occurs on the internal side of microsomes and requires UDP-GlcNAc within the vesicular lumen. Indeed, the GTP-induced synthesis of Dol-PP-oligosaccharides does not result in a correspondingly lower level of Dol-PP-GlcNAc and apparently involves a particular pool of unglycosylated dolichylphosphate (15, 17). This pool might consist of lipid molecules oriented with the phosphate group facing the lumen.

The present status of transmembrane topology in Dol-PP-oligosaccharide synthesis has been reviewed recently by Hanover and Lennarz (21) and by Snider (37). Various glycoside derivatives of dolichylpyrophosphate, in particular Dol-PP-$GlcNAc_2$, are oriented with the glycoside group facing the lumen (19, 21, 22, 38). Therefore, the question is whether the sugars, and more specially *N*-acetylglucosamine residues, cross the membrane as a nucleotide derivative (UDP-GlcNAc), as a lipid intermediate (e.g., Dol-PP-$GlcNAc_2$), or as part of a glycosylation system which receives the sugar residue from UDP-GlcNAc at the cytoplasmic surface and transfers it to the (glyco)lipid acceptor at the luminal surface. The answer it not clear yet, although the latter mechanism is presently favoured (21, 22, 37). The experimental basis of this concept is that several enzymes acting in synthesis of Dol-PP-oligosaccharides are readily inactivated by proteases in sealed microsomes (12, 21, 22, 39), and that attempts to demonstrate permeation of sugar nucleotide precursors into microsomal vesicles, or a transient cytoplasmic orientation of the chitobiosyl group of Dol-PP-$GlcNAc_2$ have failed (22). As discussed above, our results better fit a mechanism that requires transport or permeation of UDP-GlcNAc.

The stable transmembrane orientation of glycoside groups reported for the dolichylpyrophosphate derivatives is obviously consistent with glycosylation reactions being carried out at the luminal surface of the ER. The other data do not provide conclusive evidence that, in all the early steps of Dol-PP-oligosaccharide synthesis, sugar nucleotide precursors react at the cytoplasmic surface of the ER membrane. The enzyme converting dolichylphosphate into Dol-PP-GlcNAc resists proteolytic enzymes in sealed and in disrupted microsomes (22, 39, and unpublished results from A. M. Ravoet and A. Amar-Costesec). In contrast, the enzyme forming Dol-PP-$GlcNAc_2$ is inactivated by pronase treatment of intact microsomal vesicles (39). However, an enzyme may lose its activity after proteolytic cleavage at a distance from the active site, particularly when assayed in the presence of a detergent, which was the case (22, 39). Besides, the failure to demonstrate penetration of UDP-GlcNAc in systems which build up Dol-PP-oligosaccharides (possibly from preformed Dol-PP-GlcNAc; see 19, 30) without apparent alteration of the membranes is not a definite argument either. The steady state concentration of the sugar nucleotide precursor within microsomes may be noticeably lower than the concentration in the surrounding medium, and undetectable with the procedures used by Hanover and Lennarz (22).

The considerable enhancement by GTP of Dol-PP-oligosaccharide synthesis and of protein glycosylation has revealed the latency of these processes in rough microsomes from rat liver (17–19). In this respect our results are consistent with other studies reporting relatively low levels of sugar incorporation in Dol-PP-oligosaccharides and protein by untreated rough microsomes from liver (8, 31). In contrast, the hen oviduct microsomes that have been extensively studied by Lennarz and his co-workers actively assemble oligosaccharides and transfer them to protein without particular requirements (10, 30, 41). It is our contention that a latency is a meaningful fact. The apparent absence of latency in the experiments by Lennarz and co-workers (10, 30, 41) might be partially due to their use of a much higher concentration of UDP-GlcNAc, or to a greater permeability of hen oviduct membranes, or to both. The structure-dependent latency of enzymes indeed results from two distinct factors, none of which is a whole or nothing matter: the kinetics of the enzyme reaction and the rate at which the substrate can diffuse across the membrane barrier (11).

Latency of Dol-PP-oligosaccharide synthesis in liver rough microsomes and its relief by GTP in stripped membranes perhaps have other explanations than the permeability barrier of the membrane to sugar nucleotide precursors. The lateral or transmembrane freedom of movement of lipid intermediates might be greater in membranes modified by GTP. Also the concentrations of several enzymes per unit area of membrane might be so low that few individual vesicles possess the whole set of required enzymes. GTP-induced fusion, or solubilization of membrane constituents by Triton X-100, could then activate the pathway. Such assumptions cannot be ruled out, particularly because the concentration of Triton X-100 at which Dol-PP-$GlcNAc_2$ can be formed is twice that required for the release of esterase (Fig. 7) and causes extensive changes in the biochemical properties of microsomal vesicles (see Table V and reference 24) and in their morphology (24). Unfortunately, the meaning of the dissociation between the release of esterase and the synthesis of Dol-PP-$GlcNAc_2$ is blurred by the strong inhibition of *N*-acetylglucosamine incorporation into dolichylpyrophosphate derivatives which coincides with the leakage of esterase (Figs. 7 and 8). Consequently, the relatively high concentration of Triton X-100 at which Dol-PP-$GlcNAc_2$ is synthesized may merely reflect the necessity of being above the concentrations at which this inhibition occurs. The GTP-stimulated glycosylation activity did never noticeably dissociate from the permeability, which was induced without perceptible change in the biochemical properties of microsomal membranes (Table V).

The sensitivity of rough microsomes to low concentrations of GTP (Fig. 3 and reference 18) and the high specificity with respect to GTP (Table I and references 17 and 32) demon-

strate that at least one function of the ER membrane depends on this nucleotide in the cell. The major pending questions are: Which function is it? Is it restricted to the rough ER? Through which mechanism does GTP contribute to a function of the membrane? Two aspects of the changes induced by GTP in cell-free conditions are particularly puzzling: the increased permeability reported in this paper, and the condition that a least part of the ribosomes must be detached from the membrane to make rough microsomes sensitive to GTP.

It is obviously implausible that luminal constituents of ER leak out into the cytoplasm under the action of GTP, and we must conclude that membrane permeability induced in vitro is without direct meaning in the intact cell. As a consequence, we must also question the physiological significance of the other changes induced by GTP in stripped rough microsomes. This is particularly true for the dependence of core glycosylation of proteins on this nucleotide if, as envisaged above, the enhanced synthesis of Dol-PP-oligosaccharides and protein glycosylation merely reflect the free access of sugar nucleotide precursors into the vesicular lumen and result from leakiness of the membrane.

Another difficulty resides in the observation that in vitro GTP is without perceptible effect on the smooth ER-derived microsomes (Figs. 5 and 6) and acts on the rough microsomes only when a significant part of the attached ribosomes have been released from the membrane (Tables II and III and references 18 and 32). It is conceivable that GTP be involved in a membrane activity that is restricted to the rough portions of the ER and functions when ribosomes are not bound to the membrane. Such a function has not yet been convincingly established.

Another possibility is that GTP acts when ribosomes are bound to the ER and that, through their interaction with some constituents of the membrane, ribosomes hinder the process that alters the membrane barrier in cell-free systems. In these conditions the removal of ribosomes from rough microsomes would be just a way of relieving the membrane of this control, thus allowing the GTP-dependent system to manifest itself by events without direct physiological significance. Our assumption implies that a membrane function in protein biosynthesis by bound polysomes depends on GTP. It becomes plausible if, as suggested by our results, membrane effects of GTP are truly restricted to the rough microsomes. The marked increase in membrane permeability induced by GTP in vitro could reflect the uncoupling of a translocation mechanism, pointing to a function of GTP in vectorial events closely associated with the biosynthesis of glycoproteins. These prospective suggestions rest on the present experimental evidence restricting the effects of GTP to the rough elements of ER. However, since the physiological significance of the effects reported up to now are still uncertain, this restrictive character will have to be reexamined once the authentic function of the GTP at the level of the ER membrane in the intact cell is known.

The authors gratefully acknowledge the expert technical assistance of Marie-Christine Hamaide.

This work was supported by grants from the Belgian Fonds de la Recherche Fondamentale Collective and Services de Programmation de la Politique Scientifique.

Received for publication 8 October 1982, and in revised form 7 April 1983.

REFERENCES

1. Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thines-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* 61:201–212.
2. Arion, W. J., L. M. Ballas, A. J. Lange, and B. K. Wallin. 1976. Microsomal membrane permeability and the hepatic glucose-6-phosphatase system. Interactions of the system with D-mannose-6-phosphate and D-mannose. *J. Biol. Chem.* 251:4901–4907.
3. Beaufay, H., and A. Amar-Costesec. 1976. Cell fractionation techniques. *In* Methods in Membrane Biology. E. D. Korn, editor. Plenum Press, New York. 6:1–100.
4. Beaufay, H., A. Amar-Costesec, and C. de Duve. 1981. The topology of enzymes in microsomes from liver. *In* Mitochondria and Microsomes. C. P. Lee, G. Schatz, and G. Dallner, editors, Addison-Wesley Publishing Co., Reading, MA. 629–653.
5. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and H. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* 61:188–200.
6. Beaufay, H., A. Amar-Costesec, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* 61:213–231.
7. Beaufay, H., D. Godelaine, A.-M. Ravoet, and A. Amar-Costesec. 1982. GTP-induced permeability of stripped rough microsomes from rat liver. *J. Cell Biol.* 95(2, Pt. 2): 245*a*. (Abstr.)
8. Bergman, A., and G. Dallner. 1978. Incorporation of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine into proteins and lipid intermediates in microsomal and Golgi membranes from rat liver. *Biochim. Biophys. Acta.* 512:123–135.
9. Coates, S. W., T. Gurney, Jr., L. W. Sommers, M. Yeh, and C. B. Hirschberg. 1980. Subcellular localization of sugar nucleotide synthetases. *J. Biol. Chem.* 255:9225–9229.
10. Czichi, U., and W. J. Lennarz. 1977. Localization of the enzyme system for glycosylation of proteins via the lipid-linked pathway in rough endoplasmic reticulum. *J. Biol. Chem.* 252:7901–7904.
11. de Duve, C. 1965. The separation and characterization of subcellular particles. *The Harvey Lectures.* 59:49–87.
12. Eggens, I., and G. Dallner. 1980. Transverse localization of glycosyltransferases in rough and smooth microsomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 122:247–250.
13. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375–400.
14. Fleck, A., and H. N. Munro. 1962. The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. Biophys. Acta* 55:571–583.
15. Godelaine, D., and H. Beaufay. 1983. The dolichol pathway of protein glycosylation in rat liver. Evidence that GTP promotes transformation of endogenous dolichylphosphate into dolichylpyrophosphoryl-*N*-acetylglucosamine in stripped rough microsomes, *Eur. J. Biochem.* 131:667–670.
16. Godelaine, D., H. Beaufay, and M. Wibo. 1977. Incorporation of *N*-acetylglucosamine into endogenous acceptors of rough microsomes from rat liver: stimulation by GTP after treatment with pyrophosphate. *Proc. Natl. Acad. Sci. USA.* 74:1095–1099.
17. Godelaine, D., H. Beaufay, and M. Wibo. 1979. The dolichol pathway of protein glycosylation in rat liver. Incorporation of mannose into endogenous lipids and proteins of rough microsomes. *Eur. J. Biochem.* 96:27–34.
18. Godelaine, D., H. Beaufay, M. Wibo, and A. Amar-Costesec. 1979. The dolichol pathway of protein glycosylation in rat liver. Stimulation by GTP of the incorporation of *N*-acetylglucosamine in endogenous lipids and proteins of rough microsomes treated with pyrophosphate. *Eur. J. Biochem.* 96:17–26.
19. Hanover, J. A., and W. J. Lennarz. 1979. The topological orientation of *N,N'*-diacetylchitobiosylpyrophosphoryldolichol in artificial and natural membranes. *J. Biol. Chem.* 254:9237–9246.
20. Hanover, J. A., and W. J. Lennarz. 1980. N-linked glycoprotein assembly. Evidence that oligosaccharide attachment occurs within the lumen of the endoplasmic reticulum. *J. Biol. Chem.* 255:3600–3604.
21. Hanover, J. A., and W. J. Lennarz. 1981. Transmembrane assembly of membrane and secretory glycoproteins. *Arch. Biochem. Biophys.* 211:1–19.
22. Hanover, J. A., and W. J. Lennarz. 1982. Transmembrane assembly of *N*-linked glycoproteins. Studies on the topology of saccharide lipid synthesis. *J. Biol. Chem.* 257:2787–2794.
23. Katz, F. N., J. E. Rothman, V. R. Lingappa, G. Blobel, and H. F. Lodish. 1977. Membrane assembly *in vitro*: synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. *Proc. Natl. Acad. Sci. USA.* 74:3278–3282.
24. Kreibich, G., G. Ojakian, E. Rodriguez-Boulan, and D. D. Sabatini. 1982. Recovery of ribophorins and ribosomes in "inverted rough" vesicles derived from rat liver rough microsomes. *J. Cell Biol.* 93:111–121.
25. Lehle, L., and W. Tanner. 1976. The specific site of tunicamycin inhibition in the formation of dolichol-bound N-acetylglucosamine derivatives. *FEBS (Fed. Eur. Biochem. Soc. Lett.* 71:167–170.
26. Lingappa, V. R., J. R. Lingappa, R. Prasad, K. E. Ebner, and G. Blobel. 1978. Coupled cell-free synthesis, segregation, and core glycosylation of a secretory protein. *Proc. Natl. Acad. Sci. USA.* 75:2338–2342.
27. Little, J. S., D. R. Thiers, and C. C. Widnell. 1976. Latency of inosine-5'-diphosphatase in microsomes isolated from rat liver. *J. Biol. Chem.* 251:7821–7825.
28. Lodish, H. F., W. A. Braell, A. L. Schwartz, G. J. A. M. Strous, and A. Zilberstein. 1981. Synthesis and assembly of membrane and organelle proteins. *Int. Rev. Cytol.* 12 (Suppl.):247–307.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
30. Lucas, J. J., C. J. Waechter, and W. J. Lennarz. 1975. The participation of lipid-linked oligosaccharide in synthesis of membrane glycoproteins. *J. Biol. Chem.* 250:1992–2002.
31. Nilsson, O. S., M. E. De Tomas, E. Peterson, A. Bergman, G. Dallner, and F. W. Hemming. 1978. Mannosylation of endogenous proteins of rough and smooth endoplasmic reticulum and of Golgi membranes. *Eur. J. Biochem.* 89:619–628.
32. Paiement, J., H. Beaufay, and D. Godelaine. 1980. Coalescence of microsomal vesicles from rat liver: a phenomenon occurring in parallel with enhancement of the glycosylation activity during incubation of stripped rough microsomes with GTP. *J. Cell Biol.* 86:29–37.
33. Parodi, A. J., and L. F. Leloir. 1979. The role of lipid intermediates in the glycosylation of proteins in the eucaryotic cell. *Biochim. Biophys. Acta.* 559:1–73.
34. Ravoet, A. M., A. Amar-Costesec, D. Godelaine, and H. Beaufay. 1981. Quantitative

assay and subcellular distribution of enzymes acting on dolichyl phosphate in rat liver. *J. Cell Biol.* 91:679–688.
35. Rodriguez-Boulan, E., D. D. Sabatini, B. N. Pereyra, and G. Kreibich. 1978. Spatial orientation of glycoproteins in membranes of rat liver rough microsomes. II. Transmembrane disposition and characterization of glycoproteins. *J. Cell Biol.* 78:894–909.
36. Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1–22.
37. Snider, M. D. 1982. Transport of sugars during glycoprotein synthesis. *Nature (Lond).* 298:117–118.
38. Snider, M. D., and P. W. Robbins. 1982. Transmembrane organization of protein glycosylation. Mature oligosaccharide-lipid is located on the luminal side of microsomes from Chinese Hamster ovary cells. *J. Biol. Chem.* 257:6796–6801.
39. Snider, M. D., L. A. Sultzman, and P. W. Robbins. 1980. Transmembrane location of oligosaccharide-lipid synthesis in microsomal vesicles. *Cell.* 21:385–392.
40. Struck, D. K., and W. J. Lennarz. 1980. The function of saccharide-lipids in synthesis of glycoproteins. *In* The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Press, New York. 35–83.
41. Waechter, C. J., J. J. Lucas, and W. J. Lennarz. 1973. Membrane glycoproteins. I. Enzymatic synthesis of mannosyl phosphoryl polyisoprenol and its role as a mannosyl donor in glycoprotein synthesis. *J. Biol. Chem.* 248:7570–7579.
42. Wibo, M., D. Godelaine, and H. Beaufay. 1979. Incorporation of *N*-acetylglucosamine and mannose in rat liver microsomes: submicrosomal localization and effect of the removal of bound ribosomes. *In* Glycoconjugate Research. Proceedings of the Fourth International Symposium on Glycoconjugates. J. D. Gregory and R. W. Jeanloz, editors. Academic Press, Inc., New York. 2:843–845.
43. Wibo, M., D. Thines-Sempoux, A. Amar-Costesec, H. Beaufay, and D. Godelaine. 1981. Analytical study of microsomes and isolated subcellular membranes from rat liver. VIII. Subfractionation of preparations enriched with plasma membranes, outer mitochondrial membranes, or Golgi complex membranes. *J. Cell Biol.* 89:456–474.